The Reflection of the Age, Tissue and Solubility on the Amberlite CG-50 Fractions of Denatured Collagens

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Abstract

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Collagen was extracted and purified from the skins of young and full-grown guinea pigs and from the skins and tendons of calf and cow, first with neutral salt solutions and acidic buffers in the cold and then the residue was gelatinized by stepwise heating. In the young animals the collagenous material which could be solubilized at +65° C already was increased markedly. The various denatured collagens were fractionated with Amberlite CG-50 columns, eluted with buffers of increasing ionic strength and pH and finally with sodium hydroxide. The first fraction, emerging at pH 5.50, was large in the samples from soluble collagen (in comparison to insoluble), in the extracts from insoluble collagens of young animals (in comparison to adults) and in the collagenous fractions of tendon (in comparison to the skin). The gradient-buffer-eluted fraction which had been derived from insoluble collagen at progressively raised temperatures contained in abundance imino acids and hydroxy amino acids (in comparison to soluble collagens).

Denatured collagen can be divided into an arbitrary number of fractions using an Amberlite CG-50 column, eluted with buffers of increasing ionic strength and pH and finally with NaOH-solution (Kulonen *et al.* 1962). This method is suitable for the comparisons of such far-degraded gelatins, *e.g.* derived from insoluble collagens, which do not resolve to distinct fractions by carboxymethylcellulose column chromatography (Piez, Weiss and Lewis 1960, Kulonen, Virtanen and Salmenperä 1962) or by starch-gel electrophoresis (Näntö, Pikkarainen and Kulonen 1965).

The purpose of the present study was to apply this method to the comparison of collagens which differed in regard to the age and tissue of the animal or to the solubility.

Experimental

Preparation of various collagens

The skins of growing (age one week) and full-grown (age unknown) cow and of growing (weight under 500 g) and full-grown (weight 520—840 g, mean 650 g) guinea pigs were obtained immediately after killing. The isolation and purification of neutral salt-soluble (NSC)

and acid-soluble (AC) skin collagens followed the principles of Gross, Highberger and Schmitt (1955), Gallop (1955), Gross (1958) and Pikkarainen and Kulonen (1968). The temperatures in Fig. 2—4 indicate (except 120°T) the insoluble collagen (IC) dissolved at progressively raised temperatures: at 40° for 15 min, at 65°, 90° and 120° for 120 min (Pikkarainen and Kulonen 1968). The various fractions of insoluble collagen from the Achilles tendon of the calf were prepared in an analogous manner.

Fractionation by Amberlite CG-50 column chromatography

The preparative column chromatography of gelatinized collagen by Amberlite CG-50 resin has been described in detail elsewhere (Pikkarainen and Kulonen 1968). Usually 3 fractions were pooled: the pH 5.5-buffer-eluted fraction, gradient-buffer-eluted fraction and NaOH-eluted fraction (occasionally divided into 0.1 N NaOH and 1.0 N NaOH-eluted fractions).

Analytical methods

The total *content of collagen* in the various Amberlite CG-50 fractions was obtained by an integration of the absorbance values obtained by a modified biuret reaction (Pikkarainen and Kulonen 1965). Standard curves used in these calculations were made for each fraction from an acid-processed pig-skin gelatin (Eastoe 1961) dissolved in 0.1 M McIlwain's buffer (pH 5.50), in 0.5 M disodium phosphate solution, and in 0.1 N or 1.0 N NaOH.

For the determination of the *amino acid compositions* the samples were hydrolysed in 5.7 N hydrochloric acid under nitrogen in sealed tubes at +110° C for 20 h. The amino acid analyses were carried out by an amino acid analyser built according to Spackman, Stein and Moore (1958).

For the determination of the *hydroxyproline content* the samples were hydrolysed in 5.7 N hydrochloric acid at +130° C for 3 hrs. The colour reaction was carried out according to Woessner (Method II) (1961). The collagen content was calculated by multiplying the hydroxyproline content by 7.3, assuming 13.7 % of hydroxyproline in collagen.

Results

Comparison of soluble and insoluble collagens

Neutral salt-soluble and acid-soluble collagens differ from the insoluble collagen so far that the first fraction, eluted with the pH 5.5-buffer, is larger than the same fraction from insoluble collagen (Fig. 1). This difference is compensated by the large 1.0 N NaOH-eluted fraction from insoluble collagen.

The comparison was extended to the amino acid compositions of the gradient-eluted fractions both from soluble and insoluble collagens (Table I). If both amino acid values in the IC-columns deviate from the respective values in the NSC- and

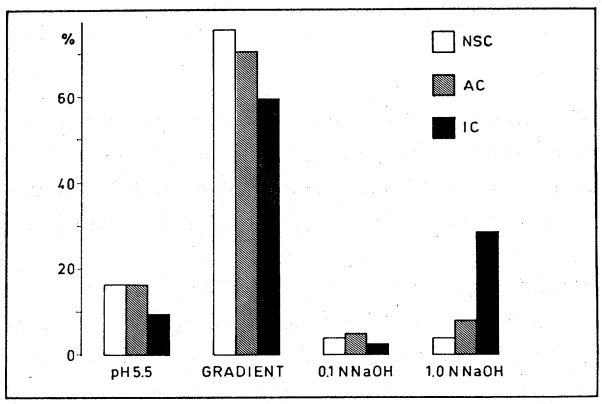

Fig. 1. Comparison of soluble and insoluble collagens. The distribution of Amberlite CG-50 fractions from gelatinized (at +120° C for 120 min) neutral salt-soluble (NSC), acid-soluble (AC) and insoluble (IC) collagens of young guinea pig skin. The ordinate indicates the percentage from total collagen.

TABLE I. Comparison of the amino acid composition of the gradient-eluted fractions from soluble and insoluble collagens of growing guinea pig skin. The Amberlite CG-50 chromatography is explained in the experimental section. The amino acid composition is expressed in residues per 1000 residues. The 0.1 N NaOH-eluted fraction of insoluble collagen is presented for comparison. NSC neutral salt-soluble collagen, AC acid-soluble collagen, IC soluble collagen. The values for serine and threonine are not corrected for the decomposition occurring during the hydrolysis and chromatography.

Amino acid	Gradient-eluted			NaOH-eluted
	NSC	AC	IC	IC
4-Hydroxyproline	96	100	111	113
Aspartic acid	49	45	48	51
Threonine	18	18	18	20
Serine	33	33	36	39
Glutamic acid	63	64	66	76
Proline	112	113	116	125
Glycine	354	352	344	350
Alanine	124	121	114	104
Valine	20	20	18	25
Methionine	4	4	6	<0.5
Isoleucine	10	11	10	11
Leucine	21	22	22	24
Tyrosine	3.	2	5	<0.5
Phenylalanine	11	13	12	12
Hydroxylysine	4	3	3	<0.5
Ornithine	1	0	0	0
Lysine	22	25	24	16
Histidine	5	3	5	0
Arginine	50	51	42	34
Acidic residues	112	109	114	127
Basic residues	82	82	74	50
Excess of acidic residues	30	27	40	77
Hydroxyproline/proline	0.86	0.88	0.96	0.90
Hydroxy amino acids	154	156	173	172
Total amino acids	208	213	227	238

AC-columns, the differences may be typical for those regions in tropocollagen, which are involved in the maturation of soluble collagens to insoluble. Such deviations are observed in both imino acids (high in IC), hydroxy amino acids (high in IC), suggestively in glutamic acid (high in IC) and in arginine (low in IC).

Comparison on the age of the animal

Insoluble collagens of guinea pig and cattle skins were studied. The effect of the age on the thermal stability is better manifested in cattle (Fig. 2). At +65° C there is a very marked difference between calf and cow in the amount of the gelatinized com-

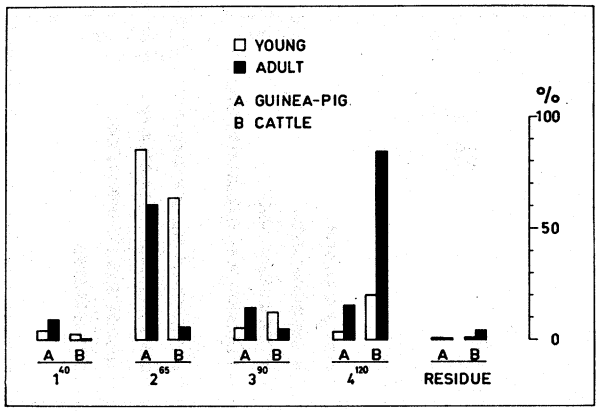

Fig. 2. Effect of the age of the animal on the thermal stability of insoluble skin collagen. The distribution of extracts obtained at stepwise heating at indicated temperatures is shown. The medium was 0.01 M acetate buffer, pH 4.8. The ordinate indicates the percentage from total insoluble collagen.

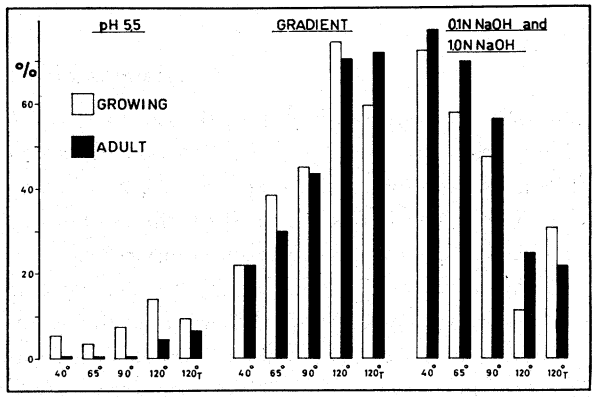

Fig. 3. The Amberlite CG-50 fractions from the various preparations of insoluble skin collagens of young and full-grown guinea pigs. The insoluble collagen (IC) was dissolved at progressively raised temperatures. 120°T means that IC was treated at 120° for 120 min without preliminary gelatinizations at lower temperatures. The ordinate as in Fig. 1.

ponents. This is reversed in the fraction which dissolves first at +120° C. Insoluble collagen of a growing animal yields relatively much of the pH 5.5-buffer-eluted fractions (Fig. 3). The NaOH-eluted fractions mirror the patterns of the pH 5.5-buffer-eluted fractions, except in the 120°T-series. During the aging insoluble collagen is so altered that after a thermal degradation it contains less of the pH 5.5-buffer-eluted and more of the NaOH-eluted component.

There were no differences in the amino acid compositions of the pH 5.5-buffer-eluted fractions of collagens from the calf and the cow.

Comparison of the skin and tendon

The insoluble collagens of the skin and tendon were chosen to represent opposite examples of the arrangement of the collagenous fibres in the tissues: the net-like and the parallel. Fig. 4 shows that there is a marked difference between the skin and the tendon in the collagenous fraction which is obtained by heating at +40° C and eluted at pH 5.5. In the distribution of the Amberlite CG-50 fractions the denatured tendon collagen resembles the behaviour of collagen from young animals.

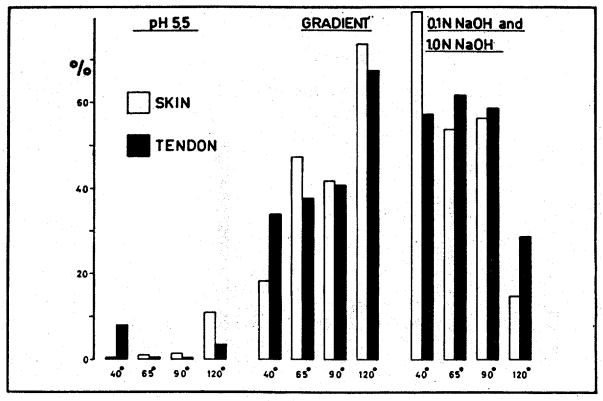

Fig. 4. Comparison of the insoluble collagens from skin and tendon. The Amberlite CG-50 fractions from the various preparations were obtained as explained in the legend of Fig. 3 and in the experimental section. The ordinate as in Fig. 1.

Discussion

The primary purpose of this work was to find from collagen soluble preparations where differences between the young and full-grown animals or between the soluble and insoluble collagens could be demonstrated. The pH 5.5-buffer-eluted and NaOH-eluted fractions obtained by the Amberlite CG-50 chromatography seem to provide suitable samples for such comparisons.

Collagen becomes less susceptible to thermal degradation at the formation of the quaternary structure, at the aging or at the formation of a network. Because the organization of collagen seems to cause a shift from pH 5.5-buffer-eluted fraction to NaOH-eluted fraction, it is relevant to ask which features in the amino acid composition are characteristic for both insoluble collagen and the NaOH-eluted fraction. There seems to be an agreement in the abundance of imino acids only (Pikkarainen and Kulonen 1968). The high content of hydroxyproline in both fractions from insoluble collagen provokes a question whether the hydroxylation of collagen proceeds during the stabilization of collagen. There are various degrees of hydroxylation of proline in collagens (Bornstein 1967a, b) from various tissues. Does the maturation affect those parts of the molecule which are rich in imino acids and probably contain an excess of acidic residues, of hydroxylated proline and of hydroxy amino acids in general? Studies on the evolution of collagen indicate that the content of imino acids has increased in higher animals, as also the thermal stability (Pikkarainen 1968).

It has been demonstrated repeatedly that the solubility of collagen decreases with advancing age: with rat-tail-tendon (Nageotte and Guyon 1934), with human skin (Bakerman 1962, Banfield 1952), with cattle and pig skin (Reich, Walther and Stather 1962), with rat skin (Mills and Bavetta 1966) and with rabbit skin (Nimni, de Guia and Bavetta 1965). The increased number of cross-links and the prevalence of larger aggregates has also been demonstrated in older animals (Heikkinen and Kulonen 1964). The present work extends this concept to the dissolution at heating,

which may be limited by similar factors as the solubility in the cold. At aging similar processes seem to continue inside the insoluble collagen, which cause its formation from the soluble precursor.

The effect of age on that proportion of insoluble collagen which can be extracted at 40° already depends on pH and other conditions (Heikkinen, E., in preparation).

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